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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE		3. REPORT TYPE AND DATES COVERED			
4. TITLE AND SUBTITLE Aminosulphydryl and Aminodisulfide Compounds Enhance Binding of the Glucocorticoid Receptor Complex to Deoxyribonucleic Acid-Coated Cellulose and to Chromatin				5. FUNDING NUMBERS			
6. AUTHOR(S) J.M. Karle, R. Olmeda and A.S. Park							
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Walter Reed Army Institute of Research Washington, DC 20307-5100				8. PERFORMING ORGANIZATION REPORT NUMBER			
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Ft. Detrick, Frederick, MD 21702-5012				10. SPONSORING/MONITORING AGENCY REPORT NUMBER			
11. SUPPLEMENTARY NOTES							
12a. DISTRIBUTION/AVAILABILITY STATEMENT APPROVED FOR PUBLIC RELEASE: DISTRIBUTION UNLIMITED						12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) In the presence of amine-containing sulphydryl compounds, binding of heat-transformed cytosolic rat liver glucocorticoid receptor complex (GRC) to double-stranded calf thymus DNA-coated cellulose and to rat liver chromatin was enhanced up to 10-fold. These observations were made under conditions when a maximum of 8% of the total GRC bound to DNA in the absence of test compound. Compounds which did not contain both a sulphydryl and amine group were inactive. Phosphorothioate derivatives of the active sulphydryl compounds were also inactive. However, pretreatment of the phosphorothioate compounds with alkaline phosphatase restored activity. Upon centrifugation at 8800g, amine-containing disulfide compounds at millimolar concentrations caused considerable sedimentation of the GRC in the absence of DNA-coated cellulose or chromatin and no apparent increase in GRC binding to DNA or chromatin. Amine-containing disulfide compounds at micromolar concentrations did not cause heavy sedimentation of the GRC and enhanced binding of the GRC to DNA-coated cellulose up to 9.5-fold. Thus, diaminosulphydryl compounds and the disulfide 1,18-diamino-6,13-diaza-9,10-dithiaoctadecane (WR 149,024) possess both the ability to restore and preserve the steroid binding capacity of the glucocorticoid receptor and to enhance binding of the GRC to DNA and chromatin.							
14. SUBJECT TERMS Glucocorticoid receptor; DNA binding; Sulphydryl; Disulfide				15. NUMBER OF PAGES			
				16. PRICE CODE			
17. SECURITY CLASSIFICATION OF REPORT		18. SECURITY CLASSIFICATION OF THIS PAGE		19. SECURITY CLASSIFICATION OF ABSTRACT			
				20. LIMITATION OF ABSTRACT			

## AMINOSULFHYDRYL AND AMINODISULFIDE COMPOUNDS ENHANCE BINDING OF THE GLUCOCORTICOID RECEPTOR COMPLEX TO DEOXYRIBONUCLEIC ACID-COATED CELLULOSE AND TO CHROMATIN

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(Received 29 March 1993; accepted 11 May 1993)

**Summary**—In the presence of amine-containing sulfhydryl compounds, binding of heat-transformed cytosolic rat liver glucocorticoid receptor complex (GRC) to double-stranded calf thymus DNA-coated cellulose and to rat liver chromatin was enhanced up to 10-fold. These observations were made under conditions when a maximum of 8% of the total GRC bound to DNA in the absence of test compound. Compounds which did not contain both a sulfhydryl and amine group were inactive. Phosphorothioate derivatives of the active sulfhydryl compounds were also inactive. However, pretreatment of the phosphorothioate compounds with alkaline phosphatase restored activity. Upon centrifugation at 8800g, amine-containing disulfide compounds at millimolar concentrations caused considerable sedimentation of the GRC in the absence of DNA-coated cellulose or chromatin and no apparent increase in GRC binding to DNA or chromatin. Amine-containing disulfide compounds at micromolar concentrations did not cause heavy sedimentation of the GRC and enhanced binding of the GRC to DNA-coated cellulose up to 9.5-fold. Thus, diaminosulfhydryl compounds and the disulfide 1,18-diamino-6,13-diaza-9,10-dithiaoctadecane (WR 149,024) possess both the ability to restore and preserve the steroid binding capacity of the glucocorticoid receptor and to enhance binding of the GRC to DNA and chromatin.

### INTRODUCTION

The effect of sulfhydryl, phosphorothioate, and disulfide compounds on the DNA binding of heat-transformed rat liver glucocorticoid receptor complex (GRC) was examined to determine if DNA binding of the heat-transformed GRC could be enhanced in the presence of these compounds. Since previous studies have shown that the DNA binding region of the rat glucocorticoid receptor is cysteine-rich [1-3] and that the GRC can only bind DNA when in a reduced form [4], the exposure of heat-transformed GRC to sulfhydryl compounds may increase the affinity of GRC binding to DNA by preserving the oxidation state of the GRC.

In the generally accepted mechanism of glucocorticoid action [3, 5], the glucocorticoid exerts its effects through a series of steps which include binding of the glucocorticoid to the

receptor to form the GRC, transformation of the GRC to a DNA binding form, and binding of the transformed GRC to glucocorticoid response elements of DNA, which results in increased transcription of specific proteins. Both the hormone binding region [6-16] and the DNA binding region [4, 9, 17-19] of the glucocorticoid receptor appear dependent upon the integrity of the sulfhydryl moieties contained in each region.

Previous studies have demonstrated that the sulfhydryl compounds dithiothreitol, glycerol, glutathione, and mercaptoethanol, are capable of preserving and/or restoring the steroid binding capacity of the glucocorticoid receptor in the presence of molybdate ion *in vitro* [8, 10, 12, 13, 16, 20]. Studies in our laboratory [21] have extended the list of protective sulfhydryl compounds. We have also demonstrated that amine-containing sulfhydryl compounds, at cellular concentrations equivalent to those obtained after oral dosing, preserve the steroid binding capacity of the glucocorticoid

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93-27243

receptor in rat hepatocytes in the absence of added molybdate ion [21].

Through cloning, sequencing, and expression of functional domains of wild-type and mutated glucocorticoid receptor proteins, the DNA binding region of the rat glucocorticoid receptor has been elucidated [1, 3, 22-24]. Eight of the 9 cysteine residues located in the DNA binding region are conserved in the receptors of the steroid and thyroid hormone superfamily and are coordinated with two zinc ions [2, 3, 23]. The zinc ions appear necessary for DNA binding of human GRC since removal of the zinc abolishes binding activity and replacement restores binding activity [2]. The importance of the integrity of the sulfhydryl moieties in the DNA binding region to binding of the GRC to DNA is shown by a number of studies. Silva and Cidlowski [4] demonstrated that the GRC can only bind DNA when in a reduced form. Young *et al.* [9] showed that the sulfhydryl-modifying agent iodoacetamide prevents binding of GRC to DNA. Bodwell *et al.* [17, 18] provided evidence that sulfhydryl groups are necessary for binding of GRC to DNA. Tienrungroj *et al.* [19] showed that rat liver cytosol GRC binding to DNA is inhibited by the sulfhydryl-modifying agent methyl methanethiosulfate and is restored after the addition of dithiothreitol.

With this study, we have established that amine-containing sulfhydryl compounds enhance DNA binding of heat-transformed rat glucocorticoid receptor to calf thymus DNA and to rat liver chromatin in a dose-dependent manner, thus adding to their ability to preserve or restore the steroid binding capacity of the glucocorticoid receptor [21]. Diaminosulfhydryl compounds are more active at enhancing GRC binding to DNA than mercaptoethylamine which contains a single amine group. Phosphorothioate derivatives of active sulfhydryl compounds enhance GRC binding to calf thymus DNA only after dephosphorylation with alkaline phosphatase. Even though

dithiothreitol, a sulfhydryl compound which does not contain amine, is a potent restorer and preserver of steroid binding capacity [8, 10, 12, 13, 16, 20, 21], dithiothreitol does not affect GRC binding to DNA. Finally, disulfide derivatives of active diaminosulfhydryl compounds also enhance binding of heat-transformed rat liver GRC to calf thymus DNA and are more potent than the diaminosulfhydryl compounds on a molar basis.

## MATERIALS AND METHODS

### Chemicals

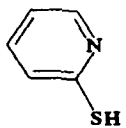
WR 347 · HCl, WR 1065 · 2HCl, WR 1729 · 2HCl, WR 2/21 · 3H<sub>2</sub>O, WR 2822 · 2H<sub>2</sub>O, WR 3689 · H<sub>2</sub>O, WR 33,278 · 2H<sub>3</sub>PO<sub>4</sub> · H<sub>2</sub>O, WR 149,024 · 4HCl, WR 151,326 · 2HCl, WR 151,327 · 3H<sub>2</sub>O, and WR 255,591 · 2HCl (Table 1) were supplied by the Division of Experimental Therapeutics, Walter Reed Army Institute of Research. All compounds were checked for purity by TLC, HPLC, and mass spectral analysis. L-Serine, L-lysine, thioglycerol, 1,4-diaminobutanone and 1,5-diaminopentane (Table 2) were purchased from Aldrich Chemical Co. (Milwaukee, WI). [6,7-<sup>3</sup>H]Triamcinolone acetonide (32.8 Ci/mmol) was purchased from Dupont/New England Nuclear (Boston, MA). Dithiothreitol, 2-mercaptopyridine, HEPES, dextran, alkaline phosphatase (from bovine intestinal mucosa, Type VII-S) and deoxyribonucleic acid-cellulose (double-stranded from calf thymus, 7 mg/g) were obtained from Sigma Chemical Co. (St Louis, MO). The charcoal (neutral Norit) and chloroacetic acid was obtained from the Fisher Scientific Co. (Fair Lawn, NJ).

2-[3-(Aminopropyl)amino][1,2-<sup>14</sup>C]ethanethiol dihydrochloride ([1,2-<sup>14</sup>C]WR 1065 · 2HCl, 55 µCi/mg) and 1,18-diamino-6,13-diaza-9,10-dithia[7,8,11,12-<sup>14</sup>C]octadecane tetrahydrochloride ([7,8,11,12-<sup>14</sup>C]WR 149,024 · 4HCl, 28.8 µCi/mg) were prepared by Dr John A. Keppler

Table 1. Chemical structures of test compounds containing both sulfur and amine groups

Sulfhydryl	Phosphorothioate	Disulfide
H <sub>2</sub> NCH <sub>2</sub> CH <sub>2</sub> SH (WR 347)		
H <sub>2</sub> N(CH <sub>2</sub> ) <sub>3</sub> NH(CH <sub>2</sub> ) <sub>3</sub> SH (WR 1065)	H <sub>2</sub> N(CH <sub>2</sub> ) <sub>3</sub> NH(CH <sub>2</sub> ) <sub>3</sub> SPO <sub>3</sub> H <sub>2</sub> (WR 2721)	[H <sub>2</sub> N(CH <sub>2</sub> ) <sub>3</sub> NH(CH <sub>2</sub> ) <sub>3</sub> S] <sub>2</sub> (WR 33,278)
H <sub>2</sub> N(CH <sub>2</sub> ) <sub>3</sub> NH(CH <sub>2</sub> ) <sub>3</sub> SH (WR 1729)	H <sub>2</sub> N(CH <sub>2</sub> ) <sub>3</sub> NH(CH <sub>2</sub> ) <sub>3</sub> SPO <sub>3</sub> H <sub>2</sub> (WR 2823)	[H <sub>2</sub> N(CH <sub>2</sub> ) <sub>3</sub> NH(CH <sub>2</sub> ) <sub>3</sub> S] <sub>2</sub> (WR 149,024)
H <sub>2</sub> CNH(CH <sub>2</sub> ) <sub>3</sub> NH(CH <sub>2</sub> ) <sub>3</sub> SH (WR 255,591)	H <sub>2</sub> CNH(CH <sub>2</sub> ) <sub>3</sub> NH(CH <sub>2</sub> ) <sub>3</sub> SPO <sub>3</sub> H <sub>2</sub> (WR 3689)	
H <sub>2</sub> CNH(CH <sub>2</sub> ) <sub>3</sub> NH(CH <sub>2</sub> ) <sub>3</sub> SH (WR 151,326)	H <sub>2</sub> CNH(CH <sub>2</sub> ) <sub>3</sub> NH(CH <sub>2</sub> ) <sub>3</sub> SPO <sub>3</sub> H <sub>2</sub> (WR 151,327)	

Table 2. Chemical structures of test compounds missing either amine groups or sulfur atoms

Lacking sulfur atoms	Lacking amine groups
$\text{HOCH}_2\text{CH}(\text{NH}_2)\text{COOH}$ (L-Serine)	$\text{HSCH}_2\text{CH}(\text{OH})\text{CH}(\text{OH})\text{CH}_2\text{SH}$ (Dithiothreitol)
$\text{H}_2\text{N}(\text{CH}_2)_4\text{CH}(\text{NH}_2)\text{COOH}$ (L-Lysine)	$\text{HOCH}_2\text{CH}(\text{OH})\text{CH}_2\text{SH}$ (Thioglycerol)
$\text{H}_2\text{N}(\text{CH}_2)_5\text{NH}_2$ (1,5-Pentanediamine)	 (2-Mercaptopyridine)
$\text{H}_2\text{NCH}_2\text{C}(=\text{O})\text{CH}_2\text{CH}_2\text{NH}_2$ (1,4-Diamino-2-butanone)	

at Research Triangle Institute (Research Triangle Park, NC) under Contract No. DAMD17-89-C-9062 to the U.S. Army Medical Research and Development Command. By TLC, these compounds were at least 95 and 97% radiochemically pure, respectively.

#### Preparation of transformed GRC

Adrenalectomized male Sprague-Dawley rats were purchased from Zivic Miller Labs (Indianapolis, IN) and were housed a minimum of 7 days prior to use. Care and feeding of the rats were performed according to the guidelines in the U.S. Department of Human and Health Services NIH Publication No. 85-23 *Guide for the Care and Use of Laboratory Animals*. Freshly excised livers from anesthetized rats (250 g, 65 mg/ml sodium pentobarbital, 0.25 ml injected i.p.) were rapidly weighed, minced with scissors, and homogenized in ice-cold 10 mM HEPES buffer (pH 7.35 at 0°C, 2 ml buffer per g of liver) with 10 strokes of a 40 ml Ten Broeck tissue grinder (Thomas Scientific, Philadelphia, PA). The homogenate was centrifuged at 27,000g for 20 min at 0–4°C, and the resulting supernatant was centrifuged at 100,000g for 1 h at 0–4°C. The supernatant was stored at –80°C until use.

The 100,000g supernatant was incubated at 0–4°C for 3 h with 50 nM [<sup>3</sup>H]triamcinolone acetone (dissolved in 10% ethanol). Final ethanol concentration was 1% [25, 26]. The ice-cold incubation solution was treated for 5 min with DNA-coated cellulose to remove any non-glucocorticoid receptor DNA binding proteins [27]. Following centrifugation for 5 min at 1500g, the resulting supernatant was treated for 15 min at 0°C with dextran-coated charcoal to remove unbound labeled steroid. The dextran-coated charcoal was pelleted at 1500g, and the supernatant was heated for 45 min at 30°C [28] in a shaking water bath.

#### DNA-coated cellulose assay

Transformed GRC solution (80 µl) was placed into triplicate 1.5-ml plastic tubes containing either 320 µl of calf thymus DNA-coated cellulose suspension (equivalent to 85 µg DNA, suspended in ice-cold 10 mM HEPES buffer, pH 7.3 at 0°C) and the test compound or 320 µl of the test compound dissolved in the HEPES buffer without the DNA-coated cellulose. Triplicate samples that contained no test compound were also included. The samples incubated at 0°C for 45 min with gentle vortexing every 15 min. Following incubation, the samples were diluted with 0.8 ml of ice-cold HEPES buffer, vortexed, and centrifuged for 2 min at 8800g. The supernatant was discarded, and the DNA-coated cellulose pellets were washed twice with 1 ml of HEPES buffer. The tip of the tube containing the DNA-coated cellulose pellet was sliced into a scintillation vial, and the radioactivity was measured using an LKB Rackbeta scintillation counter (Gaithersburg, MD). The background radioactivity which pelleted in the absence of DNA-coated cellulose was subtracted from the radioactivity found in the DNA-coated cellulose pellet.

#### Chromatin assay

Rat liver nuclei were isolated using the method of Blobel and Potter [29]. Briefly, the freshly excised rat liver was minced and homogenized as described above. The pellet from the 27,000g centrifugation was resuspended in 5 vol of ice-cold TS buffer (0.25 M sucrose and 10 mM TRIZMA BASE, pH 7.6 at 0°C), filtered through layered gauze, and centrifuged at 27,000g for 10 min. The supernatant was discarded, and the pellet was resuspended in 2.2 ml of ice-cold TS buffer. One ml of suspension was placed into two ultracentrifuge tubes. With a

syringe and a long 13 gauge needle, 2 ml of TS (KCl) buffer (1.8 M sucrose in 10 mM TRIZMA BASE containing 25 mM KCl and 3 mM  $\text{MgCl}_2$ , pH 7.6 at 0°C) was added to each tube. Following vortexing, 1 ml of TS (KCl) buffer was carefully placed at the bottom of each centrifuge tube. The tubes were centrifuged at 100,000g for 30 min. After centrifugation, the supernatant was discarded and the sides of the tubes were cleaned. Chromatin was isolated from the pellet containing the nuclei essentially as described by Simons *et al.* [30]. The nuclei were washed in 10 vol of 0.01 M TRIZMA BASE, pH 8.0 at 0°C, and were then allowed to swell in 3.5 vol of the same buffer for 2 h. The swollen nuclei were broken by sonication and centrifuged at 27,000g for 15 min. The DNA content of the supernatant layer containing the chromatin was measured by the method of Burton [31] except that propionaldehyde was used in place of acetaldehyde.

Transformed GRC solution (80  $\mu\text{l}$ ) was placed in triplicate 1.5-ml plastic tubes containing 200  $\mu\text{g}$  of chromatin (100  $\mu\text{l}$ ), and 20  $\mu\text{l}$  of either HEPES buffer or test compound dissolved in HEPES buffer. The samples were vortexed and incubated at 0°C for 45 min. Following incubation, 0.8 ml of ice-cold 10 mM HEPES buffer pH 7.35 at 0°C was added to each tube, and the samples were pelleted by centrifugation at 8800g for 4 min. The pellets were washed twice with 1 ml of the same buffer. The tip of the tube containing the chromatin pellet was sliced into a scintillation vial, and the level of radioactivity was measured. Triplicate samples with no chromatin but otherwise identical were incubated and washed simultaneously with samples containing chromatin. The background radioactivity which pelleted in the absence of chromatin was subtracted from the radioactivity found in the chromatin pellet.

#### *Dephosphorylation of phosphorothioate compounds*

Phosphorothioate compounds (0.1 M) dissolved in 10 mM HEPES buffer were incubated with alkaline phosphatase for 15 min at 37°C. The solutions were then filtered through centrifree™ ultrafiltration micropartition system (Amicon Corp., Danvers, MA). Formation of the sulfhydryl derivative of the phosphorothioate was analyzed by HPLC (Gilson, Madison, WI). The samples were eluted isocratically at 1 ml/min through a 4.6 mm  $\times$  25 cm C8 reversed phase column (5  $\mu\text{m}$  particle size, 120 Å pore

size, YMC, Inc., Morris Plains, NJ) with acetonitrile and an aqueous phase consisting of 0.1 M chloroacetic acid and 5 mM *dl*-10-camphorsulfonic acid, pH = 3.0 [32] and were detected electrochemically (BAS, West Lafayette, IN) in the oxidative mode using a glassy carbon electrode, a Ag/AgCl reference electrode, and an applied voltage of 0.95 V. The retention times for the sulfhydryl compounds WR 1065, WR 1729, and WR 255,591 were 13 to 14 min at 5% acetonitrile and for WR 151,326 was 6.4 min at 10% acetonitrile. No peaks were observed in this region for the phosphorothioate compounds.

## RESULTS

### *Enhancement of GRC binding to double-stranded calf thymus DNA-coated cellulose and rat liver chromatin by amine-containing sulfhydryl compounds*

The binding of heat-transformed rat liver GRC to double-stranded calf thymus DNA-coated cellulose and to rat liver chromatin increased up to 2.9- and 9.8-fold, respectively, in the presence of 2.5 or 5 mM concentrations of compounds containing the general formula  $\text{XN}(\text{CH}_2)_n\text{NH}(\text{CH}_2)_{n'}\text{SH}$  where  $\text{X} = \text{H}$  or  $\text{CH}_3$ ,  $n = 3$  or 5, and  $n' = 2$  or 3 (Table 3). 2-Mercaptoethylamine (WR 347) also enhanced GRC binding, but only up to 1.5-fold (Table 3). The diaminosulfhydryl compounds were also active at micromolar concentrations as shown by the enhanced binding of the GRC to the DNA-coated cellulose in the presence of 125 to 500  $\mu\text{M}$  WR 1065 and WR 255,591 (Table 4). The enhancement of GRC binding to DNA-coated cellulose and to chromatin was dose dependent at the lower test compound concentrations (Table 4). At 125 to 500  $\mu\text{M}$ , 2-mercaptoethylamine (WR 347) did not enhance GRC binding (Table 4).

The experiments were designed so that the amount of GRC binding to DNA-coated cellulose or chromatin was relatively low, only up to 8% of total GRC, in the absence of test compounds. If the amount of heated cytosol was doubled or quadrupled without increasing the amount of DNA-coated cellulose, the amount of GRC binding to the DNA-cellulose increased  $2.1 \pm 0.2$  and  $3.9 \pm 0.2$  times (mean  $\pm$  SD), respectively, for four experiments performed on separate days. No difference was observed between freshly prepared cytosol and cytosol which had been frozen. If the amount of DNA-cellulose was quadrupled relative to the

Table 3. Fold-increase in binding of heat-transformed rat liver GRC to rat liver chromatin and double-stranded calf thymus DNA-coated cellulose at millimolar test compound concentrations

Test compound	Chromatin binding <sup>a</sup>		DNA-coated cellulose binding <sup>a</sup>	
	2.5 mM <sup>b</sup>	5 mM <sup>b</sup>	2.5 mM <sup>b</sup>	5 mM <sup>b</sup>
H <sub>2</sub> NCH <sub>2</sub> CH <sub>2</sub> SH (WR 347)	1.4 ± 0.1 (4)	1.5 ± 0.1 (4)	1.2 ± 0.1 (4)	1.4 ± 0.2 (3)
H <sub>2</sub> N(CH <sub>2</sub> ) <sub>3</sub> NH(CH <sub>2</sub> ) <sub>2</sub> SH (WR 1065)	8.7 ± 3.2 (4)	6.9 ± 1.7 (7)	2.7 ± 0.6 (7)	2.0 ± 0.2 (6)
H <sub>2</sub> N(CH <sub>2</sub> ) <sub>3</sub> NH(CH <sub>2</sub> ) <sub>2</sub> SH (WR 1729)	7.8 ± 2.6 (4)	4.9 ± 1.5 (4)	1.9 ± 0.5 (5)	2.0 ± 0.5 (6)
H <sub>2</sub> CNH(CH <sub>2</sub> ) <sub>3</sub> NH(CH <sub>2</sub> ) <sub>2</sub> SH (WR 255,591)	9.2 ± 4.5 (4)	9.8 ± 4.4 (5)	2.5 ± 1.1 (8)	2.9 ± 1.3 (6)
H <sub>2</sub> CNH(CH <sub>2</sub> ) <sub>3</sub> NH(CH <sub>2</sub> ) <sub>2</sub> SH (WR 151,326)	1.9 ± 0.5 (5)	3.0 ± 1.6 (5)	2.9 ± 2.0 (5)	2.8 ± 0.5 (3)
HSCH <sub>2</sub> CH(OH)CH(OH)CH <sub>2</sub> SH (Dithiothreitol)	1.1 ± 0.1 (2)	0.9 ± 0.3 (2)	1.0 ± 0.1 (2)	1.0 ± 0.1 (4)

<sup>a</sup>Results are given as the mean ± SD of the fold-increase of the binding observed in the presence of the test compound with the number of determinations in parentheses. Each determination was performed on different days. In the absence of test compound, 0.4 to 8% of the tritium label bound to DNA-coated cellulose and 0.2 to 3% of the tritium label bound to chromatin. [<sup>3</sup>H]Triamcinolone acetonide was the steroid ligand. All values are corrected for receptor precipitation in the absence of DNA-coated cellulose or chromatin.

<sup>b</sup>Concentration of test compound.

amount of heated cytosol, the level of binding increased to 31–43% of the total amount of GRC. The GRC is being defined as the level of radioactivity of the sample following removal of the unbound radiolabeled ligand by adsorption to charcoal-coated dextran. These values are similar to literature values for the binding of rat liver GRC to DNA-coated cellulose of 36% [19], 35–60% [33], 37% [34], 11–21% [35], and 5–40% [36].

All test compounds were added to the incubations following heat-transformation of the GRC in order to ensure that the observed effects were not due to a differential amount of radiolabeled steroid bound to receptor or in the amount of transformed GRC formed in the heat-transformation step. No DNA or chromatin binding occurred when the GRC was not

transformed. The amount of carbon-14 radioactivity found associated with the DNA-coated cellulose when incubated in the presence of [<sup>14</sup>C]WR 1065 and the transformed GRC correlated with the amount of [<sup>14</sup>C]WR 1065 present relative to the amount of GRC.

Only the sulfhydryl compounds which contained a 1 or 2 amine group were found to enhance DNA or chromatin binding of heat-transformed GRC (Tables 3 and 4). Compounds with both a 1 and 2 amine group (WR 1065, WR 1729, WR 151,326, WR 255,591) were more effective than WR 347 which has only a 1 amine group. Dithiothreitol at 0.125 to 5 mM and thioglycerol at 5 mM (data not shown), two compounds known to preserve and restore the steroid binding capacity of the glucocorticoid receptor [8, 10, 12, 13, 16, 20, 21], do

Table 4. Fold-increase in binding of heat-transformed rat liver GRC to double-stranded calf thymus DNA-coated cellulose at micromolar test compound concentrations

Test compound	DNA-coated cellulose binding <sup>a</sup>			
	500 μM <sup>b</sup>	250 μM <sup>b</sup>	125 μM <sup>b</sup>	63 μM <sup>b</sup>
H <sub>2</sub> NCH <sub>2</sub> CH <sub>2</sub> SH (WR 347)	1.0 ± 0.1 (3)	0.79 ± 0.07 (3)	0.87 ± 0.04 (3)	—
H <sub>2</sub> N(CH <sub>2</sub> ) <sub>3</sub> NH(CH <sub>2</sub> ) <sub>2</sub> SH (WR 1065)	4.3 ± 0.4 (5)	2.6 ± 0.1 (5)	1.7 ± 0.1 (5)	—
H <sub>2</sub> CNH(CH <sub>2</sub> ) <sub>3</sub> NH(CH <sub>2</sub> ) <sub>2</sub> SH (WR 255,591)	4.4 ± 0.6 (3)	2.7 ± 0.2 (3)	2.0 ± 0.2 (3)	—
[H <sub>2</sub> N(CH <sub>2</sub> ) <sub>3</sub> NH(CH <sub>2</sub> ) <sub>2</sub> S] <sub>2</sub> (WR 33,278)	—	8.1 ± 1.5 (3)	4.2 ± 0.3 (3)	2.2 ± 0.1 (3)
[H <sub>2</sub> N(CH <sub>2</sub> ) <sub>3</sub> NH(CH <sub>2</sub> ) <sub>2</sub> S] <sub>2</sub> (WR 149,024)	—	9.5 ± 3.5 (4)	8.9 ± 2.0 (4)	6.0 ± 1.4 (2)
HSCH <sub>2</sub> CH(OH)CH(OH)CH <sub>2</sub> SH (Dithiothreitol)	1.2 ± 0.2 (2)	1.01 ± 0.01 (2)	1.1 ± 0.2 (2)	—
Ammonium chloride	0.84 ± 0.20 (2)	0.97 ± 0.01 (2)	0.91 ± 0.08 (2)	—

<sup>a</sup>Results are given as the mean ± SD of the fold-increase of the binding observed in the presence of the test compound with the number of determinations in parentheses. Each determination was performed on different days. In the absence of test compound, 0.7 to 8% of the tritium label bound to DNA-coated cellulose. [<sup>3</sup>H]Triamcinolone acetonide was the steroid ligand. All values are corrected for receptor precipitation in the absence of DNA-coated cellulose or chromatin.

<sup>b</sup>Concentration of test compound.

not contain an amine group and had no effect on the amount of GRC which binds to either DNA-coated cellulose or chromatin. 2-Mercaptopyridine at 5 mM was also without effect (data not shown).

*Effect of phosphorothioate derivatives of active sulfhydryl compounds*

Phosphorothioate derivatives of active aminosulfhydryl compounds, where the -SH group has been replaced by an -SPO<sub>2</sub>H<sub>2</sub> group, were ineffective in enhancing binding of the GRC to calf thymus DNA-coated cellulose. The compounds assayed were WR 2721, WR 2823, WR 151,327, and WR 3689, the phosphorothioate derivatives of WR 1065, WR 1729, WR 151,326, and WR 255,591 (Table 1), respectively. The extent of GRC binding to calf thymus DNA is unchanged in the presence of 2.5, 5, or 10 mM of the phosphorothioate compounds. Converting the phosphorothioate compound to its sulfhydryl analog with alkaline phosphatase restored its ability to enhance GRC binding to the same extent as its sulfhydryl analog. Alkaline phosphatase had no effect on GRC binding.

*Effect of disulfide analogs of selected aminosulfhydryl compounds*

The disulfide analogs of compounds WR 1065 and WR 1729 (WR 33,278 and WR 149,024, respectively) at 2.5 and 5 mM caused very high levels of sedimentation of heat-transformed GRC at 8800g and no apparent enhancement of GRC binding to DNA-coated cellulose or chromatin. Up to 50-fold more tritium sedimented in the absence of DNA-coated cellulose or chromatin than in samples containing no test compound. The high level of sedimentation was concentration dependent, diminishing below 1 mM disulfide concentration. In samples incubated with [<sup>14</sup>C]WR 149,024 in the absence DNA-coated cellulose, the amount of sedimentation of GRC correlated with the level of carbon-14 found in the sediment.

Both WR 33,278 and WR 149,024 were potent enhancers of GRC binding to DNA-coated cellulose at 63 to 250  $\mu$ M, concentrations which did not cause high rates of sedimentation of the GRC complex in the absence of the DNA-coated cellulose (Table 4). As occurred with the [<sup>14</sup>C]WR 1065, the amount of carbon-14 radioactivity found associated with the DNA-coated cellulose when incubated in the presence of [<sup>14</sup>C]WR 149,024 and the trans-

formed GRC correlated with the amount of [<sup>14</sup>C]WR 149,024 present relative to the amount of GRC. The percentage of [<sup>14</sup>C]WR 149,024 found associated with the DNA-coated cellulose was similar to the amount of [<sup>14</sup>C]WR 1065 found associated with the DNA-coated cellulose.

*Effect of amine compounds which do not contain sulfur*

At 2.5 and 5 mM concentrations of amine compounds which lack sulfur atoms, no enhancement of GRC binding to calf thymus DNA or to rat liver chromatin was observed. The compounds (Table 2) assayed included L-serine (the hydroxyl analog of L-cysteine), L-lysine, and the diamino compounds, 1,5-pentanediamine and 1,4-diamino-2-butanone, which are analogous to the diaminosulfhydryl compounds in Table 1. Ammonium chloride at 125 to 500  $\mu$ M also had no effect on GRC binding to calf thymus DNA (Table 4).

## DISCUSSION

This study shows that amine-containing sulfhydryl and disulfide compounds enhance the binding of heat-transformed rat liver GRC to double-stranded calf thymus DNA-coated cellulose and to rat liver chromatin. The compounds assayed which did not contain both an amine group and a sulfhydryl or a group convertible to a sulfhydryl were ineffective. This result correlates with the abundance of sulfhydryl and amine-containing cysteine, arginine, and lysine residues in the DNA binding domain of the rat [23], mouse [37, 38], and human [39, 40] glucocorticoid receptor. In the crystallographic structure of the DNA binding domain of the rat glucocorticoid receptor complexed with DNA [41], 8 cysteine groups form 2 tetrahedral complexes with the zinc ions maintaining the tertiary structure of the GRE. The arginine and the lysine residues form anchors to the DNA.

Recent studies [5,42] have indicated that GRC transformation is not a one-step process, but a more complex process in which the untransformed GRC first dissociates heat shock protein, then dimerizes to yield a high affinity DNA binding species. Dephosphorylation of the receptor may also be involved [43]. Thus, the mechanism by which the aminosulfhydryl and aminodisulfide compounds enhance GRC binding may be either by maintaining the DNA binding region of the glucocorticoid receptor in

a reduced basic form, by increasing the yield of the high affinity DNA binding species, or by the aminosulphydryl and aminodisulfide compounds forming a complex or covalent linkage with the GRC and increasing the number of amine groups available for interaction with the DNA. The latter possibility is consistent with the experiments with carbon-14 radiolabeled aminosulphydryl and aminodisulfide compounds which showed that the aminosulphydryl and aminodisulfide compounds bound to the DNA-coated cellulose fraction.

Although blockage of the sulphydryl moiety of the diaminosulphydryl compounds with a phosphate group destroyed the activity of the compounds, enzymatic removal of the phosphate group fully restored activity. Since the phosphate group is readily hydrolyzed by tissues [44-47], amine-containing phosphorothioate compounds have been studied clinically as a less toxic, orally absorbed prodrug of the amine-containing sulphydryl compounds [48-51].

Certain amine-containing sulphydryl compounds display both the ability to preserve the steroid binding capacity of the glucocorticoid receptor and to enhance the DNA binding of the GRC. However, the sulphydryl compounds with the highest activity for each individual property differed. The most active amine-containing sulphydryl compound in our previous study examining the capacity of the sulphydryl compound to preserve the steroid binding capacity of the glucocorticoid receptor [21] was mercaptoethylamine (WR 347), which was the least active amine-containing sulphydryl compound in the present study. In the presence of 10 mM mercaptoethylamine, the glucocorticoid receptor present in the 100,000g supernatant of rat liver homogenate retained over 80% of its binding capacity under conditions where untreated cytosolic glucocorticoid receptor retained <10% of its original binding capacity. Under the same conditions, in the presence of 10 mM of the diaminosulphydryl compound WR 151,326, the cytosolic glucocorticoid receptor retained 75% of its original steroid binding capacity. In the presence of 10 mM WR 1065, WR 1729, or WR 255,591, the cytosolic glucocorticoid receptor retained over 40% of its original steroid binding capacity. In addition, freshly isolated rat hepatocytes retained approx. 80% of the glucocorticoid receptor's steroid binding capacity in the presence of 100  $\mu$ M WR 1065 under incubation conditions that depleted

90% of the glucocorticoid receptor's binding capacity in untreated cells [21]. Both WR 151,326 and WR 1065 were shown to rapidly restore the glucocorticoid receptor's steroid binding capacity when added to glucocorticoid receptor preparations whose steroid receptor binding capacity had been depleted over 90%. Thus, the diaminosulphydryl compounds have the highest capacity to both restore/preserve the glucocorticoid receptor's steroid binding capacity and enhance GRC binding to DNA and chromatin.

The presence of disulfide forms of the diaminosulphydryl compounds at micromolar concentrations apparently did not affect the integrity of the sulphydryl moieties of the DNA binding region of the glucocorticoid receptor since enhancement of GRC binding to DNA was observed. However, unlike its sulphydryl analog, the disulfide WR 33,278 has not shown the ability to preserve the steroid binding capacity of the rat liver glucocorticoid receptor [21]. In contrast, the disulfide WR 149,024 was at least as effective as the diaminosulphydryl compounds in preserving the glucocorticoid receptor's steroid binding capacity [21].

Thus, the amine-containing sulphydryl and disulfide compounds which appear to possess both the ability to maintain glucocorticoid receptor ligand binding activity and enhance DNA binding of the GRC may augment the physiological actions and pharmaceutical properties of glucocorticoids. Kohama *et al.* [52] recently reported antiinflammatory activity for the aminodisulfides bis(2-aminoethyl) disulfide and bis(2-amino-3-phenylpropyl) disulfide administered either subcutaneously or orally. Whether the antiinflammatory activity of the disulfides is via enhancement of GRC binding to DNA and whether augmentation of GRC binding to DNA or chromatin by aminosulphydryl and aminodisulfide compounds translates into increased transcription of glucocorticoid response genes is yet to be determined.

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